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Final Report

[redacted]

Toxicity to the Water Flea *Daphnia magna* Straus under Laboratory Conditions (Acute Immobilisation Test – Semi-Static)

Data Requirements and Guidelines

OECD 202 (2004): OECD Guidelines for Testing of Chemicals No. 202. Daphnia sp., Acute Immobilisation Test. Adopted: 13 April 2004.

Study Director

[redacted]

Study Completion Date

08 May 2017

Test Facility

Eurofins Agroscience Services EcoChem GmbH / Eurofins Agroscience Services Ecotox GmbH
Eutinger Straße 24
D – 75223 Niefern-Öschelbronn
Germany

Sponsor(s)

[redacted]

EAS Study Code [redacted]

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Statement of Confidentiality

This report contains confidential and proprietary information of the sponsor, which must not be disclosed to anyone except the employees of this company or to persons authorized by law or judicial judgment without the expressed and written approval of the sponsor.

Statement of GLP Compliance

I, the undersigned, declare that the study described in this final report was conducted in accordance with the following Good Laboratory Practice (GLP) principles/regulations;

Organisation for Economic Co-operation and Development (OECD) Principles of Good Laboratory Practice and Compliance Monitoring (as revised in 1997) ENV/MC/CHEM(98)17

National regulatory guidelines/ laws were followed for the countries involved in the study. All national requirements are based on the OECD Principles of Good Laboratory Practice which are accepted by regulatory authorities throughout the European Community, the United States of America (FDA and EPA) and Japan (MHW, MAFF and METI) on the basis of intergovernmental agreements.

The study was performed according to the study plan in accordance with test facility SOP's.

Document	Date of Document
Study plan	14 Mar 2017

There were no non-compliances with respect to GLP and no deviations to the study plan (incl. amendments) likely to impact materially on the outcome of the study.

I confirm that the raw data generated to the date of the signature in the study described is valid, and this report fully and accurately reflects the procedures followed.

Date: 08 May 2017 Signature: [redacted]

Study Director

[redacted]

Acknowledgment of the Final Report

Date: 09 May 17 Signature. [redacted]

Test Facility Management

[redacted]

Quality Assurance Statement

Study code: [redacted]

Study title: [redacted] Toxicity to the Water Flea *Daphnia magna* Straus under Laboratory Conditions (Acute Immobilisation Test – Semi-Static)

This study has been audited by the relevant Quality Assurance Unit(s) in accordance with the OECD principles of Good Laboratory Practice and respective national regulations. Dates of inspection and reporting are listed in this section. Documents were audited as draft versions. Facilities and/or processes and systems are monitored as part of a regular program.

		Date of audit	Date of Report to Principal Investigator	Date of Report to Study Director[1]	Date of Report to Management[2]
Study Plan	-	30 Jan 2017	-	30 Jan 2017	30 Jan 2017
Experimental Phase	Sampling Phys.-chem. Parameter Assessment Immobilisation	17 Mar 2017	-	17 Mar 2017	17 Mar 2017
Final Report	Biological Part	24 Mar 2017	-	24 Mar 2017	24 Mar 2017
Final Report	Analytical Part	31 Mar 2017	-	31 Mar 2017	31 Mar 2017

[1] Including Lead QA and test facility management if audit reported to Principal Investigator
[2] test site management if audit reported to Principal Investigator, otherwise test facility management
- not applicable

According to the inspections detailed above, and the QA Statements provided by the test sites it can be confirmed that the methods, procedures, and observations described in this final/phase report are a full and accurate account of the raw data.

Date: 09 May 2017 Signatu[redacted]

Quality Assurance

[redacted]

Study Responsibilities

Responsibilities	Contact details	Location
Study Sponsor	[redacted]	[redacted]
Study Monitor	[redacted]	[redacted]
Test Facility	Eurofins Agroscience Services EcoChem GmbH/ Eurofins Agroscience Services Ecotox GmbH	Eutinger Str. 24 D-75223 Niefern-Öschelbronn Germany
Study Director (SD)	[redacted]	Same as test facility address

Study Schedule

Study initiation	14 Mar 2017
Experimental start	15 Mar 2017
Experimental end	29 Mar 2017
Final report	08 May 2017

Archiving and Distribution

All data and study documents to be stored at the test facility / test site will be archived in accordance with the respective SOP's of the test facility / test site.

- Archived study files and documents will be retained for a period from the issue of the final report, in accordance with the local national regulatory requirements for the test facility (currently 15 years).
- Study specific documents will be stored in the GLP Archives listed below.
- Facility-based records and documentation of QA of all sites involved will be stored in the respective GLP Archives according to the applicable national regulations.
- An aliquot of the test / reference item(s) will be retained in the dedicated archive at test facility.
- At least the following documents will be archived :

Document	**Location of GLP Archive**	**Original / Copy**
Study plan and amendments	Test Facility	Original
Study file(s) (raw data)	Test Facility	Original
Final report (and report amendments)	Test Facility	Original

At the end of the archiving period study-specific data will not be disposed of without the prior written consent of the Sponsor.

The final report was distributed as follows:

Recipient	**Original / Copy**
Study Sponsor	Original (1x), pdf file (1x)
Test Facility	Original (1x)

Corrections or additions to the final report will be made by issuing numbered report amendments.

Table of Contents

Tables

Figures

1 Summary

Report: [redacted] 2017): [redacted] Toxicity to the Water Flea *Daphnia magna* Straus under Laboratory Conditions (Acute Immobilisation Test – Semi-Static)

Source: Eurofins Agroscience Services EcoChem GmbH / Eurofins Agroscience Services Ecotox GmbH, Eutinger Str. 24, D-75223 Niefern-Öschelbronn, Germany. Unpublished report No.: [redacted] Issued: 08 May 2017.

Guidelines: OECD 202 (2004).

Deviations to Guidelines: None.

GLP: Yes (certified laboratory)

Study Objective: The objectives of this study were to determine the immobilisation effect of [redacted] on the water flea Daphnia magna under worst-case exposure conditions, the no observed effect concentration (NOEC) and the effect median concentration (EC_{50}).

Material and methods:

Test item: [redacted] batch number: [redacted]

Test species: *Daphnia magna* Straus, Clone V, max. 24 hours old.

Test design: Semi - static dose-response test with twenty organisms per test concentration (4 replicates of 5 animals each) were used. The duration of the test was 48 hours.

Endpoints: Endpoints reported are the EC_{50} and the NOEC after 24 and 48 hours.

Test rates: A semi-static main test with nominal concentrations of 10.0, 4.55, 2.07, 0.939 and 0.427 mg test item/L and control was performed.

Test conditions: Temperature, pH-value and oxygen concentration of the test solutions measured after 0, 24 hours aged and fresh and 48 hours are reported. Hardness of the test water was measured on the day of application.

Samples analysed: Analytical samples taken at 0 hours (initial value) and 24 hours from fresh and 48 h aged test solutions were analysed from control and all test item concentrations.

Statistics: The values for EC_{50} were determined by Weibull analysis using linear max. likelihood regression. The NOEC was established based on the highest concentration at which the immobilisation is not higher than the allowed control immobilisation (≤ 10 % immobilisation).

Dates of work: 15 Mar 2017 – 29 Mar 2017

Findings:

Validity criteria: Control immobilisation: The percentage of immobilisation should be ≤ 10 %. In this study the control immobilisation was 0 %.

Oxygen concentration: The dissolved oxygen concentration at the end of the test should be ≥ 3 mg/L in all test units. In this test, the dissolved oxygen concentration at the end of the test was ≥ 8.6 mg/L.

Test conditions: The total hardness (as $CaCO_3$) of the untreated control was determined to be 12°dH (214 mg/L $CaCO_3$); the mean pH-value of the untreated control was determined to be 7.88 ± 0.16 (Std. Dev.), the mean temperature of the control and all test item concentrations was measured to be 20.1 ± 0.4 °C (Std. Dev.) and the mean oxygen concentration was determined to be 8.9 ± 0.1 mg/L (Std. Dev.).

Analytical Results: The initial measured content of the [redacted] The initial measured content of the [redacted] Therefore the toxicological endpoints were evaluated using nominal and actual [redacted] concentrations.

Statistical Results: EC_{50} and NOEC-values of daphnids exposed to the test item evaluated using nominal concentrations

	[redacted] [mg/L] (nominal)	
	24 h	48h
NOEC	0.427	0.427
EC_{50}[1)]	8.00	2.68
95 % confidence limit of EC_{50}	4.91 – 20.7	1.84 – 3.62

[1)] Weibull analysis using linear max likelihood regression

EC_{50} and NOEC-values of daphnids exposed to the test item evaluated using actual concentrations based on the [redacted]

	[redacted] [mg/L] (actual*)	
	24 h	48h
NOEC	0.307	0.307
EC_{50}[1)]	5.76	1.93
95 % confidence limit of EC_{50}	3.51 – 15.9	1.32 – 2.60

[1)] Weibull analysis using linear max likelihood regression
* based on the [redacted]

Conclusions: According to the results of the test, the EC_{50} (48 h) was determined to be 2.68 mg/L (nominal) corresponding to 1.93 mg/L (actual). The corresponding NOEC (48 h) was 0.427 mg/L (nominal) corresponding to 0.307 mg/L (actual).

2 Study Objective

The objectives of this study were to determine the immobilisation effect of the [redacted] on the water flea *Daphnia magna* under worst-case exposure conditions, the no observed effect concentration (NOEC) and the effect median concentration (EC_{50}).

3 Principles of the Study

The Principles were the exposure of daphnids to test solutions and observation of immobilisation after 24 and 48 hours of exposure under semi-static conditions. The study was performed according to OECD test guideline 202 (2004).

4 Material and Methods

4.1 Test Item(s)

Test Item			
Test item name	[redacted]	Batch number	[redacted]
EAS Test item code	[redacted]	Appearance / colour	solid / magenta
Chemical name	[redacted]		
CAS number	[redacted]	[redacted]	[redacted]
Chemical formula	[redacted]	Molecular weight	855.9 to 1568.8 g/mol
Density	1.150 g/cm³	Signal word(s)	warning
Issue date of certificate	not available	Expiry date	02 Aug 2017
		Storage conditions	ambient (5 °C - 30 °C), dark, dry

Additional properties	
Water solubility	2.067 g/L

Specifications essential for correct identification of the test and reference items for use under GLP are based on information provided as by the study sponsor / supplier (e.g. certificate(s) of analysis). They have not been verified by the test facility and might have not been generated under GLP, except where this is explicitly claimed.

Additional specifications for the test item characterisation may originate from (non-GLP) sources other than the study sponsor / supplier.

4.2 Test Organism

Daphnia magna Straus, Clone V, was used as the test organism. The animals are continuously bred in the laboratory and were originally purchased in a healthy condition from the Federal Environment Agency in Berlin/Germany.

Daphnia magna was bred as single culture (1 daphnid per 100 mL) in Elendt M4 medium. The pH-value of the aerated water was within a range of 6.0 – 9.0. The dissolved oxygen was above 60 % saturation and the total hardness 140 - 250 mg/L (as $CaCO_3$), corresponding to 7.8 - 14°dH. The animals were fed with single cell green algae (*Desmodesmus subspicatus*, formerly *Scenedesmus subspicatus*) at least three times a week.

The daphnids were reared at a temperature of 20 ± 2 °C in a climatic chamber with 16 hours of illumination and 8 hours of darkness. The medium was changed three times per week. A pipette was used to separate the young daphnids from the adults.

Freshly hatched daphnids less than 24 hours old were used for the test.

4.3 Test Design

The daphnids were exposed to a range of test item concentrations and a control for 48 hours. The test concentrations were chosen based on a non-GLP range-finding test. Two concentrations of the reference item potassium dichromate (1.0 mg/L, 2.0 mg/L) were tested around the same time period as the study (see Appendix C).

4.4 Test Medium

Elendt M4 medium was used as test medium (composition see Appendix B). At test initiation the pH-value of the control (untreated test medium) was 7.82, the dissolved oxygen concentration was 9.0 mg/L and the total hardness was 12°dH (214 mg/L as $CaCO_3$).

4.5 Test Units

Glass vessel (100 mL), were filled up with ~ 50 mL test solution. The test units were covered with a glass plate (thus reducing evaporation).

4.6 Test Conditions

Test procedure:	semi-static
Duration:	48 hours
Temperature:	19.7 – 21.0 °C
Oxygen concentration:	≥ 8.6 mg/L
pH-value:	7.76 – 8.15
Exposure to light:	16 hours photoperiod /8 hours darkness daily
Feeding:	none
Test vessels:	four 100 mL glass beakers per concentration, each filled with ~ 50 mL, one additional replicate for pysico -chemical measurements without test organisms
Loading:	~ 10 mL of test solution for each animal
Aeration:	none
Number of animals:	20 per concentration in 4 replicates of 5

4.7 Application

Based on the results of a non GLP range-finding test, the following nominal concentrations were tested in the main test: 10.0, 4.55, 2.07, 0.939 and 0.427 mg test item/L and control.

The necessary amount of test item for preparing the stock solution S1 was weighed on a weighing scoop and transferred to a volumetric flask. Test medium (see Appendix B) was added up to the bench mark and the solution was homogenised by stirring for 120 minutes. A settling phase of 5 minutes was performed. Afterwards the solution was collared rose and fine particles of substance were visible. Lower test solutions were prepared by dilution of the appropriate solution with test medium. The preparation procedure was repeated after 24 hours. About 50 mL of the prepared solutions were transferred to each test vessel (see below).

Preparation of test solutions

Nominal concentration [mg/L]	Test item (required) [mg]	Dilution solution No.	Dilution solution [mL]	Final volume [mL]	Volume per test vessel [mL]	Solution No.
10.0	10.0	-	-	1000	~ 50	S1
4.55	-	S1	455	1000	~ 50	V1
2.07	-	V1	455	1000	~ 50	V2
0.939	-	V2	455	1000	~ 50	V3
0.427	-	V3	455	1000	~ 50	V4
0	-	-	-	-	~ 50	Control

4.8 Assessments

4.8.1 Biological Assessment

After 24 h and 48 h the immobilised daphnids were counted (see Appendix A: Table 1 and Table 2). All daphnids not able to swim within 15 seconds after gentle agitation of the test vessel were considered to be immobilised. If present, behavioural changes of daphnids were recorded at 24 and 48 hours after starting the test.

4.8.2 Physico- Chemical Assessment

The test temperature and the pH-value as well as the oxygen concentration of the test solutions were measured at all concentrations at t = 0 h fresh and t = 24 h from fresh test solutions and after t = 24 h and t = 48 h from aged test solutions in one separate replicate per test item concentration without test organisms (see Appendix A: Table 3 to Table 5).

4.9 Sampling and Storage

Analytical data are required by the guidelines for verification of test item concentrations as well as the stability of the test item over the entire test period. Analytical samples were taken from all test item concentrations and control at test start and after 24 hours from fresh and aged and after 48 hours from aged solutions. For each sampling also a retain sample was taken.

Samples were taken and treated as described in Appendix E (Sample Work-Up Procedure).

All samples were stored deep frozen until they were transferred to the analytical laboratory.

4.10 Chemical Analysis

The analytical verification of test item concentrations in daphnid test medium was done by analysing the content of [redacted]) in the samples during the test.

The analysis of samples was performed in the analytical laboratories of the test facility with a suitable analytical method. The results of the analysis are part of the raw data and this final report. The content of the analyts in the test solution samples was determined by analysing with [redacted] The analytical method was validated with regard to specificity, linearity, accuracy (recovery), precision and limit of quantification. Validation was performed in accordance with SANCO/3029/99 rev. 4 from 11/07/2000. The data for the analytical method and the results of the validation are represented in Appendix E.

Analytical samples were analysed from all test item concentrations and control at test start and after 24 hours from fresh solutions and after 48 hours from aged solutions. The analysed concentrations are presented in Appendix D.

4.11 Data Evaluation

The 24 h and 48 h EC_{50} are the estimated concentrations where 50 % of the daphnids were immobilised after 24 and 48 hours, respectively.

The values for EC_{50} were determined by Weibull analysis using linear max. likelihood regression. The evaluation of data was performed by ToxRat Professional 3.2.1.

The NOEC was established based on the highest concentration at which the immobilisation is not higher than the allowed control immobilisation (≤ 10 % immobilisation).

5 Results

5.1 Validity Criteria of the Study

Control immobilisation	The percentage of immobilisation should be ≤ 10 %. In this study the control immobilisation was 0 %.
Oxygen concentration	The dissolved oxygen concentration at the end of the test should be ≥ 3 mg/L in all test units. In this test, the dissolved oxygen concentration at the end of the test was ≥ 8.6 mg/L.

5.2 Biological Results

After 24 hours of exposure no immobilisation was observed in the control. No immobilisation higher than the allowed control immobilisation was observed at 0.427 mg/L. 20 % immobilisation was observed in the test item concentrations of 0.939, 2.07 and 4.55 mg/L. In the highest test item concentration of 10.0 mg/L 65 % of the daphnids were immobile. The results are presented in Appendix A: Table 1.

The daphnids were slightly collocated in the test item concentrations of 0.939 mg/L and above after 24 hours. Additionally flakes of substance were observed at the bottom of the test vessel in the test item concentrations of 4.55 and 10.0 mg/L.

After 48 hours of exposure no immobilisation was observed in the control. No immobilisation higher than the allowed control immobilisation was observed at 0.427 mg/L. 30 % immobilisation was observed at 0.939 mg/L and 35 % immobilisation was observed at 2.07 mg/L. In the concentration of 4.55 mg/L 70 % of the daphnids were immobile. At the highest test item concentration of 10.0 mg/L 95 % of the daphnids were immobile. The results are presented in Appendix A: Table 2.

After 48 hours, the daphnids were slightly collocated in the test item concentrations of 0.939 and 2.07 mg/L. The daphnids in the test item concentrations of 4.55 and 10.0 mg/L were collocated and flakes of substance were noticed at the bottom of the test vessel.

5.3 Analytical Results

The initial measured content of [redacted] The initial measured content of [redacted] Therefore the toxicological endpoints were evaluated using nominal and actual [redacted] concentrations. The analysed concentrations are presented in Appendix D.

5.4 Statistical Results

All toxicological endpoints were evaluated using nominal and actual concentrations.

EC_{50} and NOEC-values of daphnids exposed to the test item evaluated using nominal concentrations

	[redacted] [mg/L] (nominal)	
	24 h	48h
NOEC	0.427	0.427
EC_{50}[1)]	8.00	2.68
95 % confidence limit of EC_{50}	4.91 – 20.7	1.84 – 3.62

[1)] Weibull analysis using linear max likelihood regression

EC_{50} and NOEC-values of daphnids exposed to the test item evaluated using actual concentrations based on the geometric mean of the sum of the measured contents of the monosulfonic and disulfonic acid

	[redacted] [mg/L] (actual*)	
	24 h	48h
NOEC	0.307	0.307
EC_{50}[1)]	5.76	1.93
95 % confidence limit of EC_{50}	3.51 – 15.9	1.32 – 2.60

[1)] Weibull analysis using linear max likelihood regression
* based on the [redacted]

6 Conclusion

According to the results of the test, the **EC_{50} (48 h)** for immobilisation was determined to be **2.68 mg/L (nominal)** corresponding to **1.93 mg/L (actual).** The corresponding **NOEC (48 h)** was **0.427 mg/L (nominal)** corresponding to **0.307 mg/L (actual)**.

7 References

COMMISSION OF THE EUROPEAN COMMUNITIES, DIRECTORATE GENERAL FOR AGRICULTURE (1997): Storage stability of residue samples, Appendix H, 7032/VI/95 rev. 5.

EUROPEAN COMMISSION, DIRECTORATE GENERAL HEALTH AND CONSUMER PROTECTION (2000): Residues: Guidance for generating and reporting methods of analysis in support of pre-registration data requirements for Annex II (part A, Section 4) and Annex III (part A, Section 5) of Directive 91/414. SANCO/3029/99 rev. 4, 11/07/2000.

OECD (1998): OECD Principles on Good Laboratory Practice (as revised in 1997). OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring. ENV/MC/CHEM(98)17.

OECD 202 (2004): OECD Guidelines for Testing of Chemicals No. 202. *Daphnia* sp., Acute Immobilisation Test. Adopted: 13 April 2004.

TOXRAT SOLUTIONS GMBH, ToxRat Professional 3.2.1.

Appendix A

Biological Results

Table 1: Results of the test, 24 h values

	Control	0.427	0.939	2.07	4.55	10.0
		mg/L				
	immobilised daphnids after 24 h					
Group 1	0	0	2	1	1	4
Group 2	0	1	0	0	0	3
Group 3	0	0	2	3	2	3
Group 4	0	0	0	0	1	3
Σ	0	1	4	4	4	13
%	0	5	20	20	20	65

Table 2: Results of the test, 48 h values

	Control	0.427	0.939	2.07	4.55	10.0
		mg/L				
	immobilised daphnids after 48 h					
Group 1	0	0	2	2	3	5
Group 2	0	1	0	1	4	4
Group 3	0	0	3	3	4	5
Group 4	0	0	1	1	3	5
Σ	0	1	6	7	14	19
%	0	5	30	35	70	95

The temperature, pH-value and the O_2 concentration of the test solutions of the main test were measured at t = 0, 24 (fresh and aged) and 48 hours. The results are presented in Table 3 - Table 5.

Table 3: Temperature of the test solutions

	nominal test item concentration [mg/L]					
	Control	0.427	0.939	2.07	4.55	10.0
Time [h]	Temperature [°C]					
0 fresh	20.4	20.3	20.5	20.5	20.7	21.0
24 aged	19.8	19.8	19.7	19.9	19.9	19.7
24 fresh	19.8	20.0	20.2	20.3	20.5	20.8
48 aged	19.9	19.8	19.7	19.7	19.7	19.8
Mean	20.0	20.0	20.0	20.1	20.2	20.3
Std. dev.	0.3	0.2	0.4	0.4	0.5	0.7
Mean	20.1					
Std. dev.	0.4					

Table 4: pH-values of the test solutions

	nominal test item concentration [mg/L]					
	Control	0.427	0.939	2.07	4.55	10.0
Time [h]	pH					
0 fresh	7.82	7.95	7.96	7.98	7.99	7.99
24 aged	8.11	8.15	8.13	8.15	8.14	8.14
24 fresh	7.84	7.97	8.00	8.01	8.02	8.02
48 aged	7.76	8.01	8.10	8.12	8.12	8.15
Mean	7.88	8.02	8.05	8.07	8.07	8.08
Std. dev.	0.16	0.09	0.08	0.08	0.07	0.08
Mean	8.03					
Std. dev.	0.11					

Table 5: O_2 concentration of the test solutions

	nominal test item concentration [mg/L]					
	Control	0.427	0.939	2.07	4.55	10.0
Time [h]	Oxygen [mg/L]					
0 fresh	9.0	8.9	8.9	8.9	8.9	9.0
24 aged	8.9	8.8	8.8	8.8	8.8	8.8
24 fresh	9.1	9.0	9.0	9.0	9.0	9.0
48 aged	8.8	8.7	8.7	8.7	8.6	8.7
Mean	9.0	8.9	8.9	8.9	8.8	8.9
Std. dev.	0.1	0.1	0.1	0.1	0.2	0.2
Mean	8.9					
Std. dev.	0.1					

Appendix B

Composition of Test Medium

Table 6: Composition of Elendt M4 test medium

Stock solution	Concentration of stock solution		Amount (mL) of stock solution for 60 L final medium	Concentrations in final medium mg/L
Calcium chloride	$CaCl_2 \cdot 2\ H_2O$	588.16 g/L	30	294
Magnesium sulfate	$MgSO_4 \cdot 7\ H_2O$	246.6 g/L	30	123
Sodium hydrogencarbonate	$NaHCO_3$	81.0 g/L	48	64.8
Potassium chloride	KCl	11.6 g/L	30	5.80
Cation solution	$MnCl_2 \cdot 4\ H_2O$	21.63 g/L	1	0.3605
	LiCl	18.36 g/L		0.306
	RbCl	4.26 g/L		0.071
	$SrCl_2 \cdot 6\ H_2O$	9.12 g/L		0.152
	$CuCl_2 \cdot 2\ H_2O$	710.8 mg/L		0.0118
	$ZnCl_2$	780 mg/L		0.0130
	$CoCl_2 \cdot 6\ H_2O$	600 mg/L		0.0100
Anion solution	$NaNO_3$	3.29 g/L	5	0.274
	H_3BO_3	34.31 g/L		2.86
	NaBr	0.192 g/L		0.0160
	$Na_2MoO_4 \cdot 2\ H_2O$	0.738 g/L		0.0615
	KI	39 mg/L		0.00325
	Na_2SeO_3	26.3 mg/L		0.00219
	NH_4VO_3	6.9 mg/L		0.000575
Silica solution	$Na_2\ SiO_3 \cdot 9\ H_2O$	120 g/L	5	10.0
EDTA- Ferric sulphate solution	Titriplex III $\cdot 2\ H_2O$	5 g/L	30	2.50
	$FeSO_4 \cdot 7\ H_2O$	1.991 g/L		0.996
Phosphate solution	KH_2PO_4	8.58 g/L	1	0.143
	K_2HPO_4	11.04 g/L		0.184
Vitamine solution	Thiamindihydrochloride	4.5 g/L	1	0.0750
	Cyanocobalamine (B_{12})	60 mg/L		0.00100
	Biotine	45 mg/L		0.0750

Appendix C

Toxic Reference

In order to check the validity of the results, the toxicity of the reference item potassium dichromate was tested at 1.00 and 2.00 mg/L with 20 test organisms per test concentration. The results are presented in Table 7.

Table 7: Results of the toxic reference test, started on 22 Mar 2017

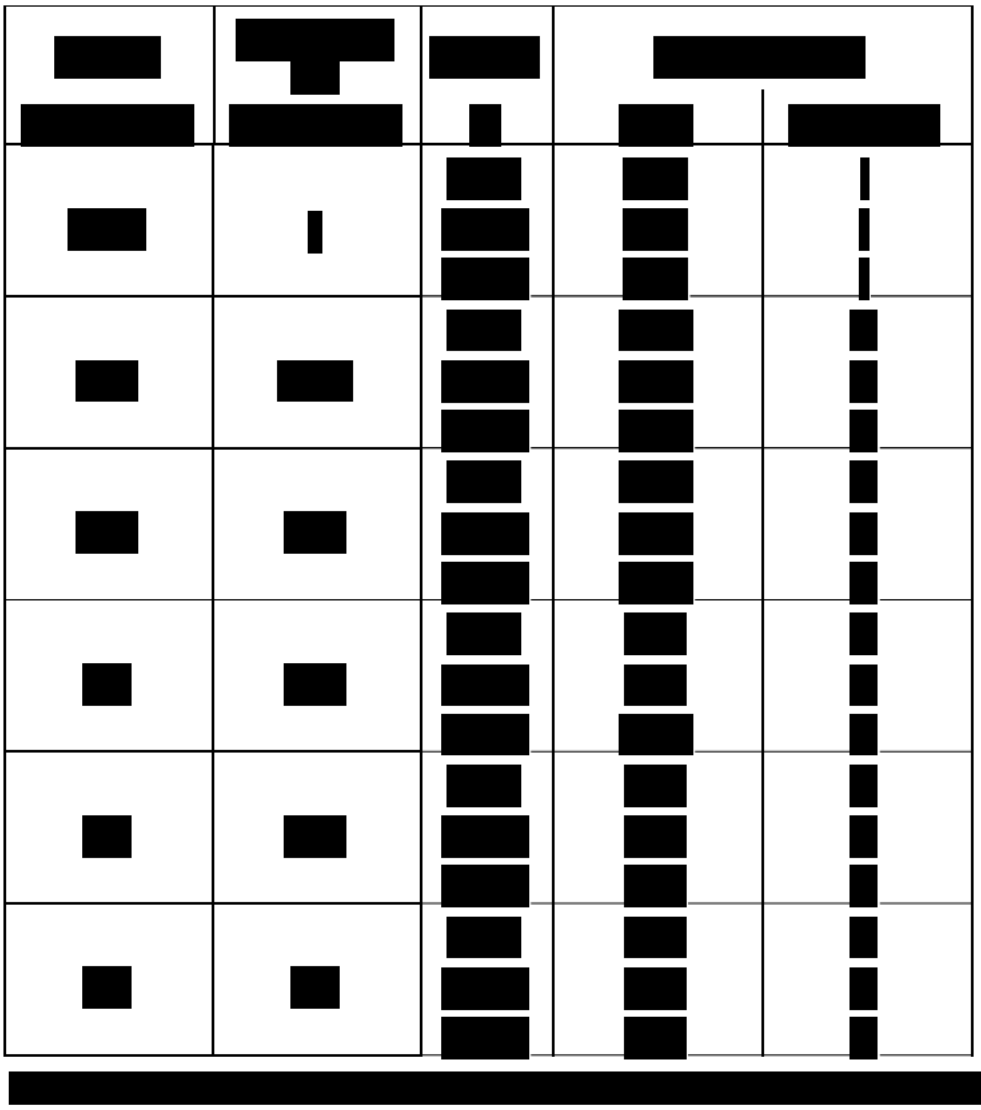
$K_2Cr_2O_7$ [mg/L]	24 h		48 h	
	1.00	2.00	1.00	2.00
	immobilised daphnids			
Group 1	2	5	4	5
Group 2	1	5	5	5
Group 3	3	5	5	5
Group 4	2	5	5	5
Σ	8	20	19	20
%	40	100	95	100

The results indicate an EC_{50} (24 h) of the reference item potassium dichromate between 1.00 and 2.00 mg/L. Since the results are in accordance with the requirements of the OECD guideline 202 and fall within the historical data generated with the reference item at the testing facility, the daphnids were suitable for the determination of the toxicological effects of the test item.

Appendix D

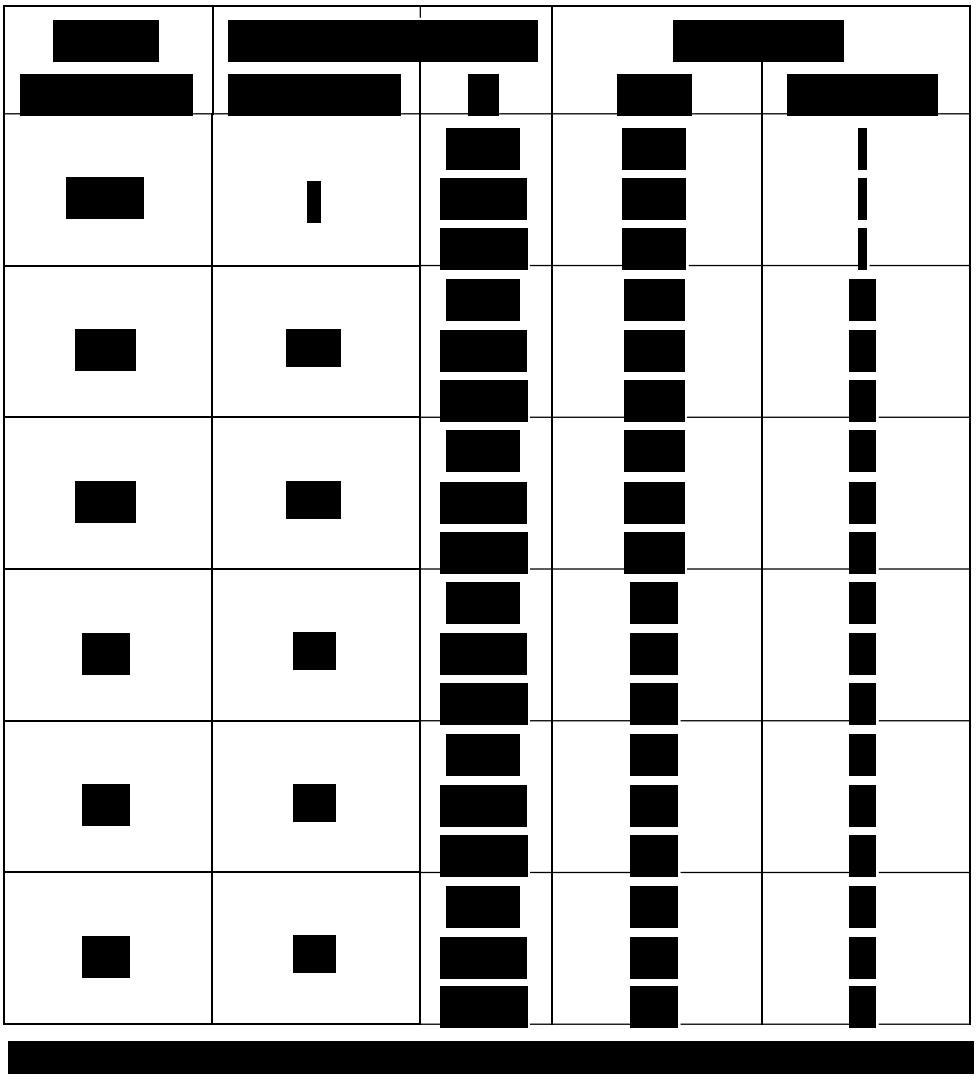
Analytical Results

[redacted]



[redacted]

[redacted]



[redacted]

Table 10: Determined concentration of the Test Item

Test item nominal [mg/L]	Sampling [h]	Test item[1)] [mg/L]	Test item[1)] % of nominal	Geometric mean [%]	Test item actual[2)] [mg/L]
Control	0 fresh	<LOD	-	-	-
	24 fresh	<LOD	-		
	48 aged	<LOD	-		
0.427	0 fresh	0.379	89	72	0.307
	24 fresh	0.303	71		
	48 aged	0.271	64		
0.939	0 fresh	0.769	82	70	0.657
	24 fresh	0.667	71		
	48 aged	0.592	63		
2.07	0 fresh	1.615	78	71	1.47
	24 fresh	1.473	71		
	48 aged	1.382	67		
4.55	0 fresh	3.699	81	76	3.46
	24 fresh	3.352	74		
	48 aged	3.405	75		
10.0	0 fresh	8.111	81	68	6.80
	24 fresh	6.768	68		
	48 aged	6.184	62		

- = not calculated; [1)] [redacted]

Appendix E

Analytical Method for the Determination of the [redacted]

An analytical method for the determination o[redacted] components in test medium was validated with regard to recovery, linearity of detector response, repeatability, specificity, limit of quantification and limit of detection. The analytical method fulfils the requirements of guideline SANCO/3029/99 rev. 4, 11/07/2000.

Specimen analysis was performed by direct injection of test medium samples and quantification by [redacted] detection.

The limit of quantification (LOQ) of the analytical method was 0.07 mg/L of test item [redacted]

The analytes were not detectable in the untreated test medium used for recovery samples. The limit of detection (LOD) was defined as 30 % of the limit of quantification [redacted]

The calibration functions were linear within the range from [redacted] with r ≥ 0.998 for both analytes, covering the working range of no more than 30 % of the LOQ to at least + 20 % of the highest analyte concentration level in a diluted sample.

The recovery was determined by fortification of untreated test medium with the test item. All mean recovery values at fortification levels of 0.07 mg/L of test item and 13 mg/L of the test item comply with the standard acceptance criteria of the guidance document SANCO/3029/99 rev 4 11/07/2000, with evaluation of one mass transition. The mean recoveries at each fortification level were in the range between 70 % and 110 % with relative standard deviations below 20 %.

Material and Methods

Test Item

A stock solution (1300 mg/L, purity not considered), and a dilution (4 mg/L) was prepared in methanol. The stock solution was used for fortification of 13 mg/L recovery samples, the dilution was used for fortification of 0.07 mg/L recovery samples.

Analytical Standard

The test item was also used as analytical standard for calibration purpose (see above). A further stock solution (1240 mg/L, purity not considered) and a dilution (1 mg/L) were prepared in methanol. Dilutions for calibration of [redacted] analysis were prepared in matrix blank extract from the 1 mg/L dilution.

Standard solution used	Volume of standard solution taken	Volume of matrix blank extract taken	Equivalent concentration	
[ng test item/mL]	[µL]	[µL]	[redacted]	[redacted]
1000	100	900	16.1	83.9
1000	70	930	11.3	58.7
1000	50	950	8.05	42.0
1000	30	970	4.83	25.2
1000	20	980	3.22	16.8
100	150	850	2.42	12.6
100	100	900	1.61	8.39

Reagents

[redacted]

Equipment

Adjustable pipettes
(Eppendorf, Brand: 10 – 100 µL, 100 – 1000 µL, 500 – 5000 µL, 1000 – 10000 µL)

Balances
(Sartorius)

Vortex-Mixer
(Scientific Industries)

[redacted]

Common laboratory glassware

Sample Work-Up Procedure

After sampling, the test medium samples (10 mL) were stored deep-frozen (≤ - 18 °C) until analysis. The samples of the timings 0h, 24h fresh and 48h aged were analysed.

In the analytical laboratory, the samples were thawed to ambient temperature and shaken using a Vortex-Mixer for 10 seconds. To the 10 mL sample, 10 mL [redacted] was added. If necessary, the samples were then diluted with blank matrix extract prior to analysis by [redacted]

Recovery samples were prepared by fortifying untreated test medium with the test item. To the 10 mL test medium 10 mL [redacted] was added. The samples were shaken on a Vortex mixer for 10 sec. If necessary, the samples were then diluted with blank matrix extract prior to analysis by [redacted].

Chromatographic and Mass Spectrometric Conditions

A summary of the chromatographic and mass spectrometric conditions used for quantification is included in the following table:

Chromatographic conditions				
[redacted]	[redacted]			
[redacted]	[redacted]			
[redacted]	[redacted]			
[redacted]	[redacted]			
[redacted]	[redacted]			
[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
	[redacted]	[redacted]	[redacted]	[redacted]
	[redacted]	[redacted]	[redacted]	[redacted]
	[redacted]	[redacted]	[redacted]	[redacted]
	[redacted]	[redacted]	[redacted]	[redacted]
	[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]			
[redacted]	[redacted]			

Mass spectrometric conditions						
[redacted]	[redacted]					
[redacted]	[redacted]					
[redacted]	[redacted]					
[redacted]	[redacted]					
[redacted]	[redacted]		[redacted]		[redacted]	
[redacted]	[redacted]		[redacted]		[redacted]	
[redacted]	[redacted]		[redacted]		[redacted]	
[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]

*used as quantifier

Specificity and Selectivity

The analytes were determined in the final specimen extracts by use of [redacted] detection.

For each analyte, one [redacted] was evaluated. A second [redacted] was monitored for confirmation of peak identity but was not used for quantification of specimens.

Untreated test medium samples were analysed according to the method to investigate the presence of residue and/or background interference at the retention time of [redacted]. The samples showed no significant interference (above 30 % of LOQ) at the retention time of analytes in any investigated test medium, therefore showing that the method is highly specific.

Linearity

The linearity of the detector response was demonstrated by single determination of matrix-matched calibration standards at six concentration levels ranging from [redacted] This range covers the range from no more than 30 % of the LOQ and at least + 20 % of the highest analyte concentration detected in any (diluted) sample.

The calibration curve was linear with correlation coefficients r ≥ 0.998 for both analytes. Linear regression was performed with 1/x-weighting.

Quantification

Quantification was performed using a calibration curve that fulfilled the above given criteria with additional correction for the mean response of standard injections bracketing the injections of the unknown specimen extracts.

If necessary, samples were diluted with blank matrix extract to be within the calibration range.

Accuracy and Precision

The method's applicability in terms of accuracy and repeatability was assessed by fortification of untreated test medium and subsequent determination of the recoveries upon applying the test method.

Five recovery determinations at 0.07 mg/L of test item (LOQ) and five recovery determinations at 13 mg/L of the test item were performed.

At least two untreated samples were analysed.

The following recoveries were obtained:

Matrix	Test Item Fortification level [mg/L]	[redacted] Nominal [mg/L]	Recovery [%]	Mean Recovery [%]	Rel. Std. Dev. [%]	Replicates
Test medium	0.07	[redacted]	92 99 98 100 90	96	5	5
	13	[redacted]	98 96 95 106 78	85	11	5

Matrix	Test Item Fortification level [mg/L]	[redacted] Nominal [mg/L]	Recovery [%]	Mean Recovery [%]	Rel. Std. Dev. [%]	Replicates
Test medium	0.07	[redacted]	103 99 96 97 87	96	6	5
	13	[redacted]	95 92 93 95 80	91	7	5

Mean recoveries and relative standard deviations per fortification level fulfil the criteria of guideline SANCO/3029/99 rev. 4, 11/07/2000 (70 – 110 % mean recovery, ≤ 20 % RSD).

In addition the following procedural recoveries were analysed:

Matrix	Test Item Fortification level [mg/L]	[redacted] Nominal [mg/L]	Recovery [%]	Mean Recovery [%]	Rel. Std. Dev. [%]	Replicates
Test medium	13	[redacted]	79 99 90	89	11	3

Matrix	Test Item Fortification level [mg/L]	[redacted] Nominal [mg/L]	Recovery [%]	Mean Recovery [%]	Rel. Std. Dev. [%]	Replicates
Test medium	13	[redacted]	95 104 98	99	5	3

Limit of Quantification and Limit of Detection

The LOQ of the method is defined as the lowest analyte concentration at which the methodology had been successfully validated. Accordingly, the LOQ of the method is 0.07 mg/L of the test item [redacted] was confirmed for [redacted] in test medium.

The LOD was set at 30 % of the LOQ which is [redacted]. As can be seen from representative chromatograms the chromatographic peaks at the LOD were equivalent to three times or more than the background noise.

Calculation of Results

The residues were calculated according to the following equation by reference to the mean response of the appropriate bracketing matrix standards as follows:

C =	$\frac{c_1 \times c_{sample} \times f_1 \times f_2}{c_2 \times 1000}$
C	Concentration in test medium sample [mg/L]
c_1	Nominal concentration of bracketing standards [ng/mL]
c_2	Average calculated concentration of standards bracketed between samples, obtained from the calibration function [ng/mL]
c_{sample}	Analysed concentration of the sample, as calculated from the calibration function [ng/mL]
f_1	Dilution factor at laboratory (10 mL sample + 10 mL [redacted] = 19.5 mL final volume, dilution factor 19.5/10.5 = 1.95)
f_2	Dilution factor before analysis
1000	Conversion from ng/mL to mg/L

Recovery rates were calculated by the following equation:

Rec =	$\frac{C \times 100\%}{C_{nominal}}$
Rec	Recovery [%]
C	Concentration determined [mg/L]
$C_{nominal}$	Fortified concentration [mg/L]

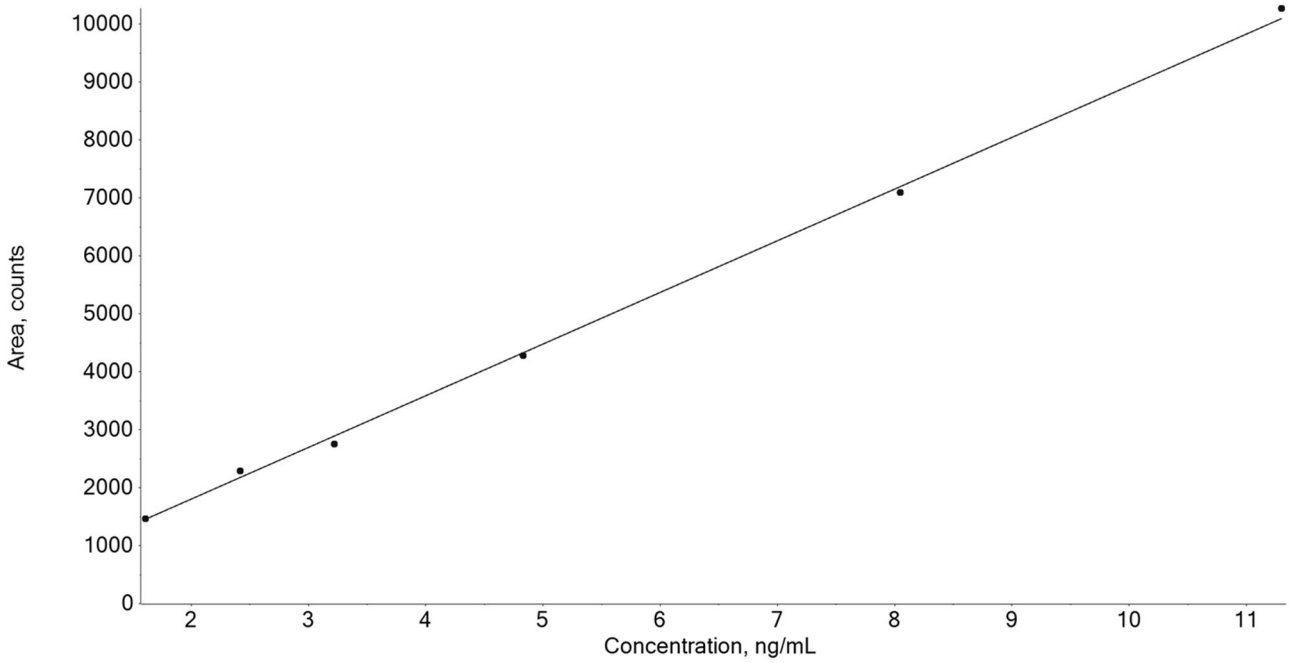
Storage Stability

The maximum storage period from sampling to analysis was 8 days within this study. Residues are regarded as stable if the samples are stored deep-frozen for up to 30 days between sampling and analysis (EU COM 7032/VI/95). Therefore, the storage stability o[redacted] was not verified.

Appendix F

Calibration Data and Chromatograms

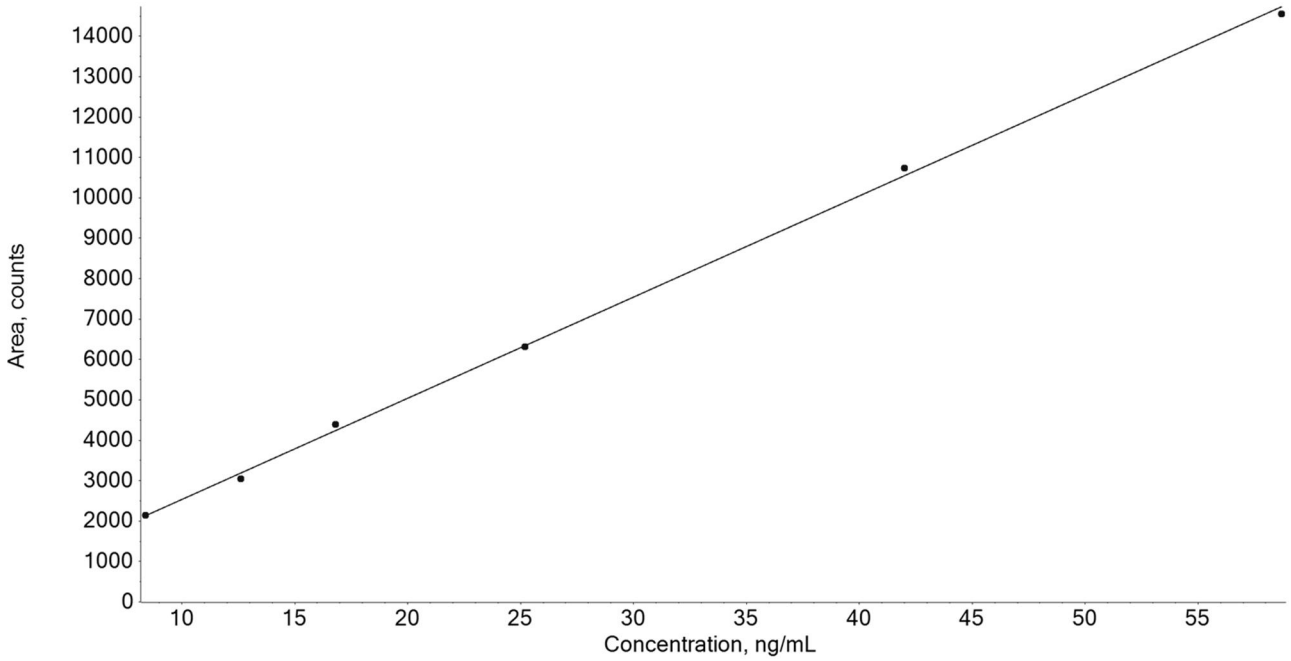
y = 892 x + 19.9 (r = 0.9991)



Nominal concentration [ng/mL]	Peak area	Calculated concentration [ng/mL]
11.3	10270	11.5
8.05	7094	7.93
4.83	4276	4.77
3.22	2751	3.06
2.42	2287	2.54
1.61	1465	1.62

Figure 1: Typical calibration curve – [redacted]

y = 250 x + 31.4 (r = 0.9994)



Nominal concentration [ng/mL]	Peak area	Calculated concentration [ng/mL]
58.7	14550	98.8
42.0	10729	102
25.2	6312	99.5
16.8	4388	104
12.6	3047	95.6
8.39	2150	101

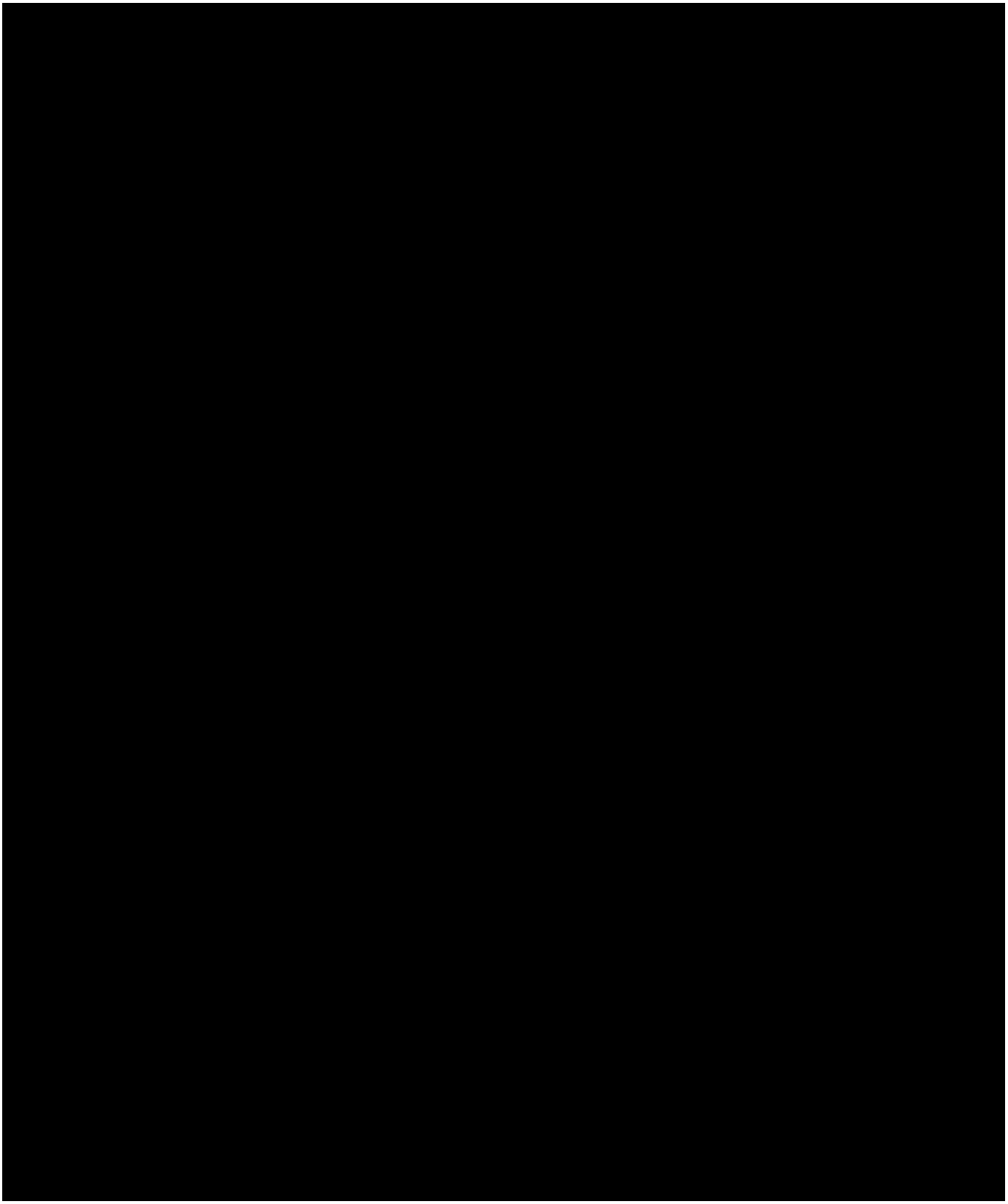
Figure 2: Typical calibration curve – [redacted]

[redacted]

[redacted]

[redacted]

[redacted]



[redacted]

Appendix G

GLP Certificate of Test Facility

Baden-Württemberg

LANDESANSTALT FÜR UMWELT, MESSUNGEN UND NATURSCHUTZ BADEN-WÜRTTEMBERG

Gute Laborpraxis / Good Laboratory Practice

GLP-Bescheinigung / Statement of GLP Compliance

(gemäß / according to § 19 b Chemikaliengesetz)

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 2004/9/EG wurde durchgeführt in:

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 2004/9/EC at:

☒ Prüfeinrichtung / Test facility ☐ Prüfstandort / Test site

Eurofins Agroscience Services EcoChem GmbH / Eurofins Agroscience Services Ecotox GmbH

Eutinger Straße 24

75223 Niefern-Öschelbronn

(Unverwechselbare Bezeichnung und Adresse / Unequivocal name and adress)

Prüfungen nach Kategorien / Areas of Expertise

(gemäß / according ChemVwV-GLP Nr. 5.3 / OECD guidance)

1	**Prüfungen zur Bestimmung der physikalisch-chemischen Eigenschaften**	**Physical-chemical testing**
4	**Ökotoxikologische Prüfungen zur Bestimmung der Auswirkungen auf aquatische und terrestrische Organismen**	**Environmental toxicity studies on aquatic and terrestrial organisms**
5	**Prüfungen zum Verhalten im Boden, im Wasser und in der Luft; Prüfungen zur Bioakkumulation und zur Metabolisierung**	**Studies on behavior in water, soil and air; bioaccumulation**
6	**Prüfungen zur Bestimmung von Rückständen**	**Residue studies**
7	**Prüfungen zur Bestimmung der Auswirkungen auf Mesokosmen und natürliche Ökosysteme**	**Studies on effects on mesocosms and natural ecosystems**
8	**Analytische Prüfungen an biologischen Materialien**	**Analytical and clinical chemistry testing**

Datum der Inspektion / Date of Inspection

(Tag.Monat.Jahr / day.month.year)

10.10.2013

Die/Der genannte Prüfeinrichtung/Prüfstandort befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.
Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung/diesem Prüfstandort die oben genannten Prüfungen unter Einhaltung der GLP-Grundsätze durchgeführt werden können.

The above mentioned test facility/test site is included in the national GLP Compliance Programme and is inspected on a regular basis.

Based on the inspection report it can be confirmed, that this test facility/test site is able to conduct the aforementioned studies in compliance with the Principles of GLP.

Unterschrift, Datum / Signature, Date

[redacted]

Karlsruhe, 10.12.2015

(Name und Funktion der verantwortlichen Person / Name and function of responsible person)

LUBW Landesanstalt für Umwelt, Messungen und Naturschutz Baden-Württemberg
Postfach 10 01 63, 76231 Karlsruhe

(Name und Adresse der GLP-Überwachungsbehörde / Name and address of GLP Monitoring Authority)